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(54) Title: EXPRESSION OF INFLUENZA NUCLEOPROTEIN ANTIGENS IN BACULOVIRUS (57) Abstract <p>The present invention relates to nucleoprotein (NP) genes of the influenza A and B viruses which were constructed from virion RNA and subsequently expressed in <i>Spodoptera frugiperda</i> (S19) cells using the baculovirus vector, <i>Autographa californica</i> nuclear polyhedrosis virus (AcNPV). The baculovirus-expressed NP antigens were reactive with monoclonal antibodies specific for either type A or B NP and with anti-NP antibodies in human serum samples. The expressed influenza NP antigens comigrated in electrophoresis with NP purified from influenza A or B virions and the recombinant NP antigens represented greater than 10 % of total protein in infected cells. Dilutions of clarified S19 cell lysates were used as antigens in a standard enzyme immunoassay format to detect serum antibody specific for influenza A or B viruses. The results showed that baculovirus-expressed NP antigens can be used in reproducible and routine assays for the serodiagnosis of influenza virus infections.</p>		

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EXPRESSION OF INFLUENZA NUCLEOPROTEIN ANTIGENS IN BACULOVIRUS

BACKGROUND OF THE INVENTION

Field of the Invention

5 The present invention relates to baculovirus-expressed influenza antigens, in particular, to the influenza A and B viral nucleoprotein antigens expressed from Autographa californica nuclear polyhedrosis virus (AcNPV). The invention further
10 relates to bioassays for the detection of influenza viral infections and to the use of such proteins in vaccines against influenza A or B virus.

Background Information

 The nucleoprotein (NP) is the major protein
15 component of the ribonucleoprotein core of influenza virions and is a type-specific antigen defining influenza viruses as either type A or type B. Recent studies have also identified the nucleoprotein antigen as a major target of the cross-reactive cytotoxic T-
20 cell response against influenza viruses (Townsend et al., 1984, Journal of Experimental Medicine 160, 552-583); therefore, cellular immunity to NP may contribute to recovery from influenza infection (Wraith et al., 1987, Journal of General Virology 68, 433-440).

25 As antigenic variation of NPs occurs more slowly than does that of influenza surface glycoproteins (Van Wyke et al., 1980, Journal of Virology 35, 24-30), the NP is particularly useful for serodiagnostic tests for the detection of influenza infections in mammals. The
30 test for measuring antibodies to NP has traditionally been a complement fixation (CF) test. However, the CF test has several drawbacks. For example, the CF test requires that a large number of biological reagents be standardized. In addition, the NP antigen preparations

used in the CF test are likely to contain other virus-specific antigens. Serodiagnostic tests which overcome these drawbacks would be valuable tools for the detection of influenza infections.

5 Since the NP antigen is antigenically conserved, it is an ideal candidate for expression by recombinant DNA methodology as part of the development of a new generation of diagnostic assays for influenza virus infections. Viral antigens produced by recombinant DNA
10 expression systems can provide an inexhaustible source of chemically defined material for use in serodiagnostic assays, experimental vaccines, and fundamental research. These techniques also eliminate the costs and potential hazards in the large-scale
15 cultivation of pathogenic viruses. For example, the use of baculovirus-expressed Hantaan virus nucleoprotein as a diagnostic antigen has been reported recently (Schmalijohn et al., 1988, Journal of General Virology 69, 777-786). Further, the use of NP antigen
20 produced in Escherichia coli (E. coli) in an experimental diagnostic assay for influenza A has been reported (Harmon et al., 1989, Journal of Medical Virology 24, 25-30). This assay was found to be superior to CF for influenza virus diagnosis.

25 The recently developed eucaryotic expression system using recombinant baculovirus, Autographa californica nuclear polyhedrosis virus (AcNPV), should be useful for producing antigens for immunoassays for the serologic diagnosis of viral infections (Luckow et
30 al., 1987, Bio/Technology 6, 47-55). Infecting insect cells (Spodoptera frugiperda) with such recombinant baculoviruses allows for the production of large amounts of antigen (Possee, R.D., 1986, Virus Research

5, 43-59). In addition, the baculovirus system has other important advantages over the commonly used methods of producing viral antigens. For example, with the baculovirus system the viral antigens are produced in cells that do not contain antigens that cross-react with antibodies in most human serum. Therefore, the purification of the antigen that is required for proteins expressed in bacterial and yeast systems may not be required with baculovirus-expressed antigens. Further, baculoviruses do not infect humans and can therefore be safely handled in large quantities.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a less costly, easier and safer means of producing influenza A and B NP antigens than provided with previous methods.

It is another object of the present invention to provide baculovirus-expressed nucleoproteins from influenza A and B viruses for use in serodiagnostic assays for influenza virus.

It is a further object of the present invention to provide serodiagnostic assays for the detection and diagnosis of influenza A and B viral infections.

Various other objects and advantages of the present invention will become apparent from the drawings and the following detailed description of the invention.

In one embodiment, the present invention relates to a DNA construct comprising a DNA segment encoding a nucleoprotein of influenza A or influenza B virus; and a vector comprising a polyhedrin gene promoter from a baculovirus, baculovirus flanking sequences and a bacterial origin of replication. The DNA segment of

the construct is operably linked to the polyhedrin gene promoter of the vector.

In another embodiment, the present invention relates to recombinant baculoviruses and to host cells infected therewith. Recombinant baculoviruses to which the present invention relates encode a nucleoprotein of influenza A or influenza B virus. Host insect cells of the present invention are infected with a recombinant baculovirus in a manner allowing the expression of the NP encoded in the baculovirus.

In another embodiment, the present invention relates to baculovirus-expressed influenza A or influenza B virus nucleoprotein and to a method of producing the baculovirus-expressed influenza A or B virus nucleoprotein. Baculovirus-expressed influenza A or B virus nucleoproteins are produced by culturing host insect cells of the present invention, in a manner allowing the expression of the nucleoprotein encoded in the infecting baculovirus, and isolating the nucleoprotein from the cells.

In a further embodiment, the present invention relates to a bioassay for the diagnosis of influenza in mammals comprising the steps of coating a surface with the baculovirus-expressed nucleoprotein of the present invention, contacting the coated surface with a biological sample from a mammal suspected of having influenza, and detecting the presence or absence of a complex formed between the protein and antibodies specific therefore present in the biological sample. The present invention also relates to diagnostic kits comprising the baculovirus-expressed nucleoprotein and ancillary reagents suitable for use in detecting the

presence or absence of antibodies to the protein in a biological sample.

In yet a further embodiment, the present invention relates to a vaccine for mammals against influenza A or
5 influenza B virus. The vaccine comprises the baculovirus-expressed nucleoprotein of the present invention, in an amount sufficient to induce immunization against the virus, and a pharmaceutically acceptable carrier.

10 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the construction scheme of baculovirus transfer vectors. Solid black lines indicate plasmid DNA and black lines with dashed inner lines indicate AcNPV DNA. Abbreviations are S: SmaI,
15 P: PvuII, B: BamHI, Sa: Sall, E: EcoRI, X: Xba I, CIP: calf intestinal phosphatase, MBN: mung bean nuclease. Arrows indicate the start site of AcNPV polyhedrin gene mRNA and binding regions of the B1 and B10 sequencing primers.

20 Figure 2 demonstrates detection of baculovirus-expressed influenza A or B NP by fluorescent antibody. Sf9 cells were infected with AcANP (A), AcBNP (C) or wild type AcNPV (B) fixed and stained with FITC-conjugated monoclonal antibody to type A NP (A, B) or
25 type B NP (C, P). Magnification was 400x.

Figure 3 demonstrates the detection of influenza A or B NP genes in recombinant baculovirus. DNA from pANP (A) or pBNP (B) (lane 1), AcANP or AcBNP (lane 2) and wild type AcNPV (lane 3) was digested with Sall and
30 EcoRI (A) or BamHI and PvuII (B) separated by agarose gel electrophoresis, transferred to nitrocellulose and hybridized to ³²P-labeled primers specific for either A NP (A) or B NP (B).

Figure 4 shows a Western blot analysis of baculovirus-expressed influenza A or B NP antigens. Samples are purified influenza A (A) or B (B) virions (lane 1) or lysates of cells infected with wild-type AcNPV (lane 4), AcANP (A2, B3), and AcBNP (B2, A3).
5 Samples were electroblotted after electrophoresis and hybridized to monoclonal antibodies specific for A NP (A) or B NP (B).

Figure 5. Panel A shows Coomassie Blue-stained gel from Figure 4. Lanes 1 and 2 are lysates of Sf9
10 cells infected with AcANP (1) or AcBNP (2); lane 3 contains molecular-weight markers. Bands identified as either A or B NP by immunological staining are marked. Panel B shows spectrodensitometric scans of the gels
15 from panel A.

Figure 6 demonstrates the reactivity of postinfection mouse serum with baculovirus-expressed influenza A or B NP antigens by enzyme immunoassay (EIA). The horizontal axis is serum dilution and the
20 vertical axis is O.D. units after EIA. Open squares represent normal mouse serum, filled squares represent serum from mice infected with type B influenza, and crosses represent serum from mice infected with type A influenza.

Figure 7 shows comparison titers obtained in EIA assays using baculovirus-expressed NPs compared to titers obtained using bacterially-expressed ANP or CF. Panel A shows comparison of EIA titers using bacterial (y axis) or baculovirus-expressed (x axis) ANP for 24
25 serum samples (Table 1, adults 1-7, and 8-13). Panels B and C show comparison of EIA titers (x axis) using baculovirus-expressed ANP (panel B, 24 points, Table 1: adults 1-7, and 8-13) or BNP (panel C, 18 points, Table
30

1: adults 4-7, and 8-13) NP antigens with titers obtained in CF assays (y axis). Coefficient of correction(r)=0.88(A), 0.58(B), 0.83(C). Level of significance was 0.01 for all panels.

5 Figure 8 shows Western blot reactivity of human serum with baculovirus-expressed influenza NP antigens. Lysates of Sf9 cells infected with AcANP (left lanes) or AcBNP (right lanes) were subjected to electrophoresis and transferred to nitrocellulose and
10 hybridized to pairs of acute-phase (S1) and convalescent-phase (S2) human serum specimens that had increased antibody reactivity specifically to either influenza A (A1:Table 1, adult 1, A2:Table 1, adult 5) or influenza B (B:Table 1, adult 8) or to respiratory
15 syncytial virus (C:Table 1, adult 14).

 Figure 9 shows Western blot reactivity of serum from infant children with baculovirus-expressed influenza A and B NP antigens. Acute and convalescent serum pairs (S1 and S2) were from an individual
20 receiving monovalent influenza type A vaccine (A:Table 1, child 3), having a natural influenza type A infection (B:Table 1, child 1) or from a child infected with parainfluenza virus (C:Table 1, child 5). Lysate containing baculovirus-expressed ANP was run in right
25 lane, BNP was run in left lane as in Figure 8.

DETAILED DESCRIPTION OF THE INVENTION

 The present invention relates to a system for expressing influenza A and B NP antigens utilizing baculoviruses and to the proteins made therewith. NP
30 proteins to which the present invention relates react specifically with anti-influenza NP antibodies and, therefore, can be used in serodiagnostic assays. The present invention provides an easier and safer means of

producing influenza virus NPs and is less costly than current methods. For example, the present invention is safer than the isolation of NP from whole virus as the recombinant viruses of the present invention are not
5 infectious for mammals. In addition, the NP antigens of the present invention do not need further costly purification in order to separate them from other proteins which react strongly with human serum. Such separation is necessary for NP antigens produced in
10 bacterial systems.

In one embodiment, the present invention relates to a DNA construct encoding a NP antigen of an influenza A or B virus. The DNA construct comprises a DNA segment encoding a NP of an influenza A or
15 influenza B virus and a vector. The vector comprises the polyhedrin gene promoter region of a baculovirus, the baculovirus flanking sequences necessary for proper cross-over during recombination (the flanking sequences comprise about 200-300 base pairs adjacent to the
20 promoter sequence) and a bacterial origin of replication which permits the construct to replicate in bacteria. The vector is constructed so that (i) the DNA segment is placed adjacent (or operably linked or "downstream" or "under the control of") to the
25 polyhedrin gene promoter and (ii) the promoter-NP antigen combination is flanked on both sides by 200-300 base pairs of baculovirus DNA (the flanking sequences). Suitable vectors for use in the present invention include, but are not limited to, pACYM1.

30 To produce the DNA construct of the present invention, a cDNA clone encoding the full length NP of an influenza A or influenza B virus is obtained using methods known in the art and inserted into a plasmid,

such as, for example, pUC 8. Then plasmid containing the NP gene is then ligated into a transfer vector, for example, pAcYM1, to yield the DNA construct.

The DNA construct of the present invention is used
5 to generate recombinant baculoviruses. The DNA
construct is contacted in a host cell with baculovirus
DNA of an appropriate baculovirus (that is, of the same
species of baculovirus as the promoter encoded in the
construct) under conditions such that recombination is
10 effected. The resulting recombinant baculoviruses
encode either the full length influenza A virus NP or
influenza B virus NP. For example, an insect cell can
be cotransfected or transfected separately with a DNA
construct of the present invention and a functional
15 baculovirus. Resulting recombinant baculoviruses can
then be used to infect insect cells to effect
production of the NP protein.

In another embodiment, the present invention
relates to host insect cells, for example, Spodoptera
20 frugiperda cells, producing a baculovirus-expressed
influenza NP. Insect host cells infected with a
recombinant baculovirus of the present invention and
cultured under conditions allowing expression of the
baculovirus-encoded NP produce baculovirus-expressed NP
25 antigens. NPs thus produced are then isolated from the
cells using methods known in the art. Further, it is
contemplated that host cells can be stably transformed
with the DNA constructs of the present invention.

The baculovirus-expressed influenza nucleoproteins
30 of the present invention may be more reactive than the
previously described bacterially-expressed ANP (Harmon
et al., 1989, Journal of Medical Virology 24, 25-30).
The bacterially-expressed antigen is a fusion protein

which contains part of a bacterial protein. The baculovirus-expressed NPs are complete copies of the viral NP genes and no additional amino acids have been added. The bacterially-expressed ANP showed decreased reactivity compared to viral NP on Western blot analysis. Unexpectedly, no decrease in reactivity was observed when the baculovirus-expressed NPs were analyzed in the same manner. Accordingly, the NP antigens of the present invention should provide a more reactive material for use in bioassays and vaccines to detect and prevent influenza A and/or B viral infections.

In another embodiment, the present invention relates to bioassays for the diagnosis of influenza virus infections in mammals. Using standard diagnostic protocols the baculovirus-expressed nucleoproteins of the present invention can be used to detect the presence of antibodies specific therefore in biological samples without undue experimentation.

For example, by coating a solid surface such as a polystyrene microtitration plate, a slide or nylon or nitrocellulose membranes generated by immunoblot or Western blot, with the baculovirus-expressed NP of the present invention and contacting the surface with a biological sample, such as serum, the presence or absence of antibodies to the influenza A or B virus can be detected. If antibodies are present in the sample formation of antibody-protein complexes is effected. These complexes can be detected using standard methodologies known in the art. As the NP of the present invention appears more reactive than the bacterially-produced NP smaller amounts may be needed

in the bioassay. Further, the bioassays may be more accurate due to this additional reactivity.

For a general diagnosis of an influenza infection the solid surface is coated with both the influenza A NP and influenza B NP of the present invention. For the determination of a specific type of influenza infection (that is, an influenza A or B viral infection), the solid surface is coated with either the influenza A NP or the influenza B NP of the present invention.

The present invention further relates to diagnostic kits. The diagnostic kits of the present invention comprise the baculovirus-expressed NP antigens of the present invention and ancillary reagents suitable for use in detecting the presence or absence of antibodies or the DNA constructs of the present invention necessary for production of the NP and ancillary reagents.

In a further embodiment, the present invention relates to vaccines for mammals against influenza A or influenza B viral infections. Protective antibodies against influenza can be raised by administering to a mammal a vaccine comprising the baculovirus expressed nucleoproteins of the present invention in a pharmaceutically acceptable carrier. The baculovirus expressed NP antigen is present in the vaccine in an amount sufficient to induce immunization against the virus. As one skilled in the art will understand, it is not necessary to use the entire protein. A unique, antigenic portion of the influenza nucleoprotein can be used. Vaccines of the present invention can also include effective amounts of immunological adjuvants known to enhance an immune response.

The following examples are given to further illustrate the present invention without being deemed limitative thereof.

EXAMPLES

- 5 cdNA cloning of influenza type A and B NP genes.
Virion RNA was extracted from gradient-purified influenza B/Ann Arbor/1/86 and A/Ann Arbor/6/60 (wild-type) viruses by standard methods (Cox et al., 1983, Bulletin of the World Health Organization 61, 143-152).
- 10 cdNA copies of total viral RNA were prepared by the method of Lapeyre and Amairic (Lapeyre et al., 1985, Gene 37, 215-220) except that first-strand synthesis by reverse transcriptase was primed by using universal influenza type A or B primers complementary to the 3'
- 15 untranslated region of virion RNA. The double-stranded cdNA fragment corresponding to influenza genomic RNA segment 5 (influenza A: 1565 base pairs; influenza B: 1811 base pairs) was isolated from agarose gels, purified, and ligated into the Sma I site of plasmid
- 20 pUC 8 using standard methods (Maniatis et al., 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) Bacterial colonies (E. coli, HB101) containing recombinant plasmids with NP inserts were identified by in situ
- 25 hybridization (Maniatis et al., (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) to ³²P-labeled, oligonucleotide primers with sequences specific for influenza A or B NP genes.
- 30 The size, sequence, and orientation of the inserted NP genes were determined by a rapid plasmid-sequencing technique (Chen et al., 1985, DNA 4, 165-171) using the M13 forward and reverse primers and by

restriction endonuclease analysis. (The complete nucleotide sequences of both NP genes have been published see Cox et al., 1983, Bulletin of the World Health Organization 61, 143-152 and Rota, P.A., 1989, 5 Nucleic Acids Research 17, 3595). Plasmids containing full-length cDNA copies of the influenza A and B NP genes were designated pANP and pBNP, respectively (Fig. 1). The A NP cDNA clone was 1565 bases in length; the B NP was 1811. Both clones contained the entire NP 10 coding sequence and 5' untranslated region, and both NP genes were oriented in the pUC plasmids with the 5' end of the gene closest to the BamHI site on the pUC polylinker (Fig. 1).

15 Expressions of influenza nucleoproteins in insect cells.

Influenza A and B NP genes were excised from pANP and pBNP by restriction endonuclease digestion, purified by agarose gel electrophoresis, made blunt-ended by treatment with mung bean nuclease, and ligated 20 into the Sma 1 site of the transfer vector, pACYM1B1 (Fig. 1). pACYM1B1, a derivative of pACYM1 (Matsuura et al., 1987, Journal of General Virology 68, 1233-1250) contains a multiple cloning site inserted at the unique Bam HI site of pACYM1 (gift of Anthony Sanchez, 25 Centers for Disease Control, Atlanta, GA).

Transformant colonies containing the inserted NP genes were identified by in situ hybridization as mentioned earlier. The proper orientation of the NP genes with respect to the AcNPV polyhedrin gene 30 promoter was confirmed by sequencing purified plasmid DNA using oligonucleotide primers, B1 and B10 (also a gift of Anthony Sanchez, Centers for Disease Control, Atlanta, GA.), designed to sequence the 5' and 3'

termini of DNA fragments inserted into the multiple cloning site of pACYM1B1 (Fig. 1).

The sequences also indicated that the translation start sites of the A and B NPs were, respectively, 50
5 and 58 bases downstream from the deleted translation start site on the AcNPV polyhedrin gene (Matsuura et al., 1987, Journal of General Virology 68, 1233-1250) and therefore 100 and 108 bases downstream of the transcription start site of the AcNPV polyhedrin gene
10 mRNA.

Insect cell culture and propagation of AcNPV were done by using previously described methods (Summers et al., 1987, Texas Agricultural Experiment Station Bulletin No. 1555). Purified pYM1ANP and pYM1BNP DNA
15 were each cotransfected with purified AcNPV DNA into *Spodoptera frugiperda* (Sf9, ATCC CRL 1711) cells according to the protocol described by (Summers et al., 1987, Texas Agricultural Experiment Station Bulletin No. 1555).

20 After 5 to 6 days of incubation, serial dilutions (10^{-3} to 10^{-8}) of the culture supernatants were prepared in Hink's medium, and 20 ul amounts of each dilution were inoculated into Sf9 cells that had been seeded into 96-well tissue-culture plates. The supernatants
25 from each well, harvested after 5 to 6 days, were transferred to a sterile 96-well plate for storage and the remaining cells were resuspended in 100 ul of phosphate-buffered saline (PBS), lysed by freezing and thawing, and spotted onto nitrocellulose filters.

30 Cultures expressing influenza A or B NP were detected by incubating the filters with either influenza A or B NP-specific monoclonal antibodies (Walls et al., 1986, Journal of Clinical Microbiology

23, 240-245). Hybridization and wash conditions were identical to those used for Western blot analysis (Tsang et al., 1983, Methods in Enzymology 92, 377-391) except that bound antibody was visualized with
5 ^{125}I -Staphylococcal protein A. Recombinant viruses expressing either the influenza A or B NP were purified from cultures that were strongly positive for antigen expression by three successive rounds of plaque isolation (Summers et al., 1987, Texas Agricultural
10 Experiment Station Bulletin No. 1555). Only plaques that were negative for polyhedrin gene expression by light microscopic examination were selected.

Sf9 cells infected with the recombinant viruses were removed from the culture vessel, washed with
15 Grace's medium, fixed onto microscope slides with ice-cold acetone and examined by fluorescent antibody assay using fluorescein-isothiocyanate (FITC)-conjugated influenza A or B NP-specific monoclonal antibodies (Boots-Celltech, Berks, U.K.) (Walls et al., 1986,
20 Journal of Clinical Microbiology 23, 240-245). Evaluation of the baculovirus recombinant with a fluorescent antibody assay was repeated after plaque purification of the recombinant viruses, AcANP and AcBNP (Fig. 2). Cells infected with purified
25 recombinant baculovirus showed intense, granular fluorescence when stained with FITC-conjugated monoclonal antibodies specific for either influenza A or B NP. In some cases large, positively staining aggregates were visible in the cytoplasm of the
30 infected cells. Sf9 cells infected with wild-type AcNPV gave little background fluorescence.

Analysis of recombinant baculoviruses.

DNA from the plaque-purified recombinant baculoviruses, AcANP and AcBNP, was extracted from the cytoplasm of infected Sf9 cells and analyzed by
5 restriction endonuclease digestion and Southern blot hybridization by standard procedure (Maniatis et al. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

The Southern analyses of the purified DNA from Sf9
10 cells is shown in Figure 3. Restriction fragments that comigrated with NP genes excised from the pUC plasmids and also hybridized to influenza A or B NP-specific ³²P-labeled oligonucleotide probes were detected in the blots of restriction digests of DNA from AcANP and
15 AcBNP. Therefore the recombinant baculoviruses appeared to contain complete copies of the appropriate NP genes.

Lysates prepared from infected cells were subjected to electrophoresis on 10% polyacrylamide gels
20 containing 4M urea (Lamb et al., 1978, Virology 91, 60-78) followed by Western blot analysis (Tsang et al., 1983, Methods in Enzymology 92, 377-391). To quantitate the amount of influenza A or B NP contained in cell lysates, gels containing electrophoretic
25 separations were stained with Coomassie brilliant blue and the amount of NP protein present determined by scanning densitometry.

Western blot analysis of total cell lysates prepared from Sf9 cells infected with either the A or
30 the B NP-producing recombinant virus indicated that the NP antigens being expressed by the recombinant viruses were recognized by influenza A or B NP-specific monoclonal antibodies and had electrophoretic

mobilities that were identical to those of NPs contained in purified influenza A or B virions (Fig. 4). The monoclonal antibodies did not cross-react with any other antigens contained in Sf9 cell lysate or with the NP antigen of the heterologous subtype. In this gel system, the A NP migrated with an apparent molecular weight of approximately 53 kilodaltons while the B NP was slightly larger, approximately 58 kilodaltons (Fig. 5). Electrophoretic separations of lysates from infected Sf9 cells were also stained with Coomassie brilliant blue and analyzed by scanning densitometry (Fig. 5). The protein bands that had been identified in the Western blot as either A or B NP protein were found to comprise approximately 10% to 20% of the total protein in the lysate.

The results of a time-course study done by infecting cells in stationary culture at a multiplicity of infection of 5 pfu/cell and calculating the amount of NP contained in total cell lysates by Western blot indicated that the level of expression for the B NP was greater than that of the A NP. In this experiment, peak production of both NPs occurred 4 to 5 days postinoculation and reached a maximum of 200 to 300 ug NP/ 10^8 cells for the BNP and 50-100 ug NP/ 10^8 cells for the ANP.

The level of expression of the type A NP was lower than that of the type B NP, even though both genes were inserted into the transfer vector in similar orientations. The influenza-specific sequences immediately upstream from the NP initiation codons are different in type A and B viruses and could have different effects on expression levels. Optimum levels of expression have been obtained when the intervening

sequences were removed and the initiation codon of the expressed antigen was placed as close as possible to the deleted polyhedrin protein initiation codon on the transfer vector (Matsuura et al., 1987, Journal of General Virology 68, 1233-1250).

Antigenic reactivity of baculovirus-expressed NP antigens.

Postinfection mouse serum was prepared by infecting groups of six A/J mice intranasally with 10^5 TCID₅₀ of either influenza B/England/222/82 or A/Ann Arbor/6/60. Serum samples were obtained from each group at 14 days postinfection and pooled.

Adult human serum samples used in this study were from the same set as was used in a previous study with bacterially expressed influenza A NP (Harmon, et al., 1989, Journal of Medical Virology 27, 25-30). These serum samples had been obtained during the acute and convalescent stages of illness of patients from whom influenza A or B viruses were isolated. All of these serum pairs used had rises in antibody titer to either influenza A or B detected by hemagglutination inhibition and CF (see Table 1 below, adults 1-13). Serum pairs from infant children which had natural influenza infections as documented by virus isolation (see Table 1 below, children 1 and 2) and from infant children receiving monovalent influenza A vaccine (see Table 1 below, children 3 and 4) were also used. Paired serum samples from adults and infant children with rises in antibody titer to other respiratory viruses (see Table 1 below, adults 14 and 15, children 5 and 6) as well as normal human infant serum (see Table 1 below, children 7 and 8) and normal chimpanzee serum were used as negative controls.

To prepare antigens for EIA assay, Sf9 cells were infected with the recombinant AcNPVs, and the cells were harvested when the cytopathic effect was advanced (usually 4-5 days). The cells were pelleted, washed
5 with Hink's medium, and resuspended in PBS with 1M NaCl. The cells were disrupted by freeze-thawing followed by Dounce homogenization and centrifuged for 1 hour at 10,000 x g to pellet cellular debris. Electrophoretic analysis of pellet and supernatant
10 fractions revealed that most of the NP antigen was present in the supernatant.

Lysates of cells infected with a recombinant baculovirus expressing the Lassa fever virus glycoprotein gene (gift of David Auperin and Kimberly
15 Hummel, Centers for Disease Control, Atlanta, GA) were prepared as described above and used as negative control antigens.

For EIA, the supernatants were diluted in PBS containing 1% fetal bovine serum and coated onto 96-
20 well polystyrene plates for 24 hours at room temperature. The optimal concentration of the lysates used for coating the plates was a 1:1000 dilution of the BNP lysate and a 1:500 dilution of the ANP lysate containing respectively 3 and 6 μ g of protein/ml
25 (approximately 30 ng NP/well) and was determined in assays using the NP-specific monoclonal antibodies to detect bound NP. Wash buffer was PBS plus 0.5% Tween 20. After washing, the plates were preincubated for 1 hour with PBS, 0.5% Tween, plus 1% fetal bovine serum.
30 Dilutions of the serum specimens were then added and allowed to incubate for 2 hours at room temperature. The plates were washed three times, and bound antibody was detected by using Staphylococcal protein A

conjugated to horseradish peroxidase with o-phenylene-
diamine and hydrogen peroxide as substrate. Titers
were expressed as the reciprocal of the highest serum
dilution giving a reading of greater than .100 in the
5 EIA. Titers listed in Table 1 are the average of
duplicate tests on each samples.

Figure 6 shows the reactivity of the antigens with
serum samples from mice infected with either type A or
B influenza. Normal mouse serum showed little
10 background reactivity with either of the test antigens.
Serum samples obtained from mice 14 days after
infection with influenza virus reacted strongly with
the NP antigen of the same virus type used for the
infection but not with the NP antigen preparation from
15 the heterologous influenza type. These results
indicated that the NP antigens were antigenically
reactive when coated onto the EIA plates and that the
remainder of the proteins present in the lysate were
causing little background reactivity.

20 The EIA was then tested with a series of acute-
(S1) and convalescent-phase (S2) adult human serum
pairs that had demonstrated four-fold rises in titer
against either influenza A or B as determined by both
CF and by hemagglutination inhibition. Table 1 shows
25 that the EIA using the baculovirus-expressed antigens
detected serum antibody rises specific for either type
A or B influenza.

The titers obtained using the baculovirus-
expressed ANP antigen showed excellent correlation with
30 titers obtained in assays using highly purified
bacterially-expressed ANP (Fig. 7A). The titers
obtained using the baculovirus-expressed A and B NP
antigens showed a strong positive correlation with

titers obtained using the traditional CF assay (Fig. 7B). All of the adult serum pairs that had a four-fold rise in antibody titer against influenza A as detected by CF (Table 1, Adults 1-7) also had a four fold rise
5 in titer when tested with the baculovirus-expressed ANP by EIA.

The EIA using the baculovirus-expressed BNP was slightly more sensitive than CF and for three of the five serum pairs tested detected greater fold rise in
10 antibody titer than did the CF assay (Table 1, Adults 8-13). Also, it appeared that the EIA using the baculovirus-expressed antigens was more sensitive than the CF assay for detecting low levels of antibody in acute serum samples. Of the baculovirus-expressed
15 antigens, the B NP was generally more reactive and gave higher titers than the A NP, even though equivalent amounts of the two antigens were seeded onto the EIA plates.

Many of the acute serum samples from adults
20 reacted both with A and B NP antigens as would be expected for human serum. Control serum pairs from adults that had antibody rises to other respiratory viruses (respiratory syncytial virus and parainfluenza) also showed some reactivity with the influenza A NP
25 antigen. However, eight acute phase or normal serum samples from infant children and one sample from a chimpanzee gave no background reactivity with the baculovirus antigen preparations (Table 1, children 1-8). In addition, EIA plates prepared using lysates of
30 Sf9 cells infected with a polyhedrin-minus recombinant AcNPV showed little background reactivity with any of the human serum samples (Table 1). A polyhedrin-minus baculovirus recombinant was used for the control in

these experiments rather than wild-type AcNPV because other investigators have shown that human serum binds nonspecifically to the very abundant AcNPV polyhedrin protein. Therefore, the reactivity of the human acute
5 serum samples from adults was most likely due to pre-existing antibody to influenza NP antigens from prior exposure to or vaccination with influenza rather than by non-specific reactivity of the human serum to baculovirus or Sf9 cell antigens.

10 To further investigate the reactivity of human serum with the baculovirus-expressed influenza NP antigens, lysates of Sf9 cells infected with the recombinant baculoviruses were subjected to SDS-polyacrylamide gel electrophoresis, blotted onto
15 nitrocellulose, and hybridized to several of the human acute- and convalescent-phase (S1 and S2) serum pairs (at 1:200 dilutions) from Table 1.

The Western blot in Figure 8 confirmed that all the acute serum samples from adults contained
20 antibodies that reacted with NP antigens. In serum pairs that had a previously determined rise in antibody titer to influenza type A, the amount of antibody to the A NP in the convalescent-phase (S2) serum increased visibly relative to the acute-phase serum (S1); the
25 amount of antibody that recognized the B NP remained constant for both serum samples. The reverse was true for the serum pair in which there was a rise in antibody titer to influenza B. The control samples also had detectable levels of NP antibody, but no
30 increase in anti-NP activity was apparent between the acute- and the convalescent-phase serum samples.

Acute and control serum samples from infant children showed no reactivity with the baculovirus-

expressed NP antigens in Western blot assays (Fig. 9).
These results confirm that the background from the
adult acute serum samples was due to pre-existing
antibody and showed that human serum did not react with
5 any of the other proteins contained in these crude
baculovirus antigen preparations.

All publication mentioned hereinabove are hereby
incorporated by reference.

While the foregoing invention has been described
10 in some detail for purposes of clarity and
understanding, it will be appreciated by one skilled in
the art from reading of this disclosure that various
changes in form and detail can be made without
departing from the true scope of the invention.

Table 1. Comparison of the reactivity of human serum samples with baculovirus-expressed influenza NP antigens with other standard diagnostic tests.

serum	EIA titer when tested with				Complement fixation titer	
	Baculovirus-expressed		Bacterially-expressed		Influenza A	Influenza B
	ANP	BNP	Control	ANP ^a		
Rises to influenza A						
Adult 1	800 3200	3200 3200	100 100	2560 10240	<16 32	
Adult 2	400 1600	3200 1600	100 100	640 2560	<8 16	
Adult 3	400 1600	200 400	100 100	2560 10240	16 64	
Adult 4	1600 6400	800 1600	100 100	10240 40960	8 32	8 8
Adult 5	1600 6400	1600 1600	100 100	10240 40960	8 32	8 <32
Adult 6	1600 6400	1600 3200	100 100	10240 40960	<16 32	<8 <16
Adult 7	1600 6400	400 400	100 100	10240 40960	8 32	<8 <8
Child 1	200 1600	<100 <100	<100 <100			
Child 2	<100 800	<100 <100	<100 <100			
Child 3 ^a	200 3200	<100 <100	<100 <100			
Child 4 ^a	<100 400	<100 <100	<100 <100			

24.1

Rises to influenza B							
Adult 8	S1	1600	200	100	2560	<8	<8
	S2	1600	25600	100	2560	<8	128
Adult 9	S1	800	400	200	2560	<8	<8
	S2	800	6400	100	2560	<32	64
Adult 10	S1	400	800	100	640	<8	<8
	S2	800	6400	100	640	<16	16
Adult 12	S1	1600	400	100	2560	8	<8
	S2	1600	25600	100	2560	8	512
Adult 13	S1	1600	100	100	2560	<16	<16
	S2	1600	6400	100	2560	<16	32
Rises to other respiratory viruses							
Adult 14	S1	800	100	100			
	S2	800	200	100			
Adult 15	S1	800	100	200			
	S2	800	100	200			
Child 5	S1	<100	100	100			
	S2	<100	100	100			
Child 6	S1	<100	<100	<100			
	S2	<100	<100	<100			
Normal serum							
Child 7		<100	<100	<100			
Child 8		<100	<100	<100			
Chimpanzee		<100	<100	<100			

a. titers from Harmon et al., 1989.
b. monovalent influenza vaccine recipients.

WHAT IS CLAIMED IS:

1. A DNA construct comprising:
 - i) a DNA segment encoding a nucleoprotein of influenza A or influenza B virus; and
 - 5 ii) a vector comprising a polyhedrin gene promoter from a baculovirus, baculovirus flanking sequences and a bacterial origin of replication, wherein said DNA segment is operably linked to the polyhedrin gene promoter of said vector.
- 10 2. The DNA construct according to claim 1 wherein said baculovirus promoter is Autographa californica nuclear polyhedrosis virus promoter.
3. The DNA construct according to claim 1 wherein said vector is pACYM1B1.
- 15 4. A recombinant baculovirus encoding a nucleoprotein of influenza A or influenza B virus.
5. An insect cell infected with said recombinant baculovirus according to claim 4 in a manner allowing the expression of said nucleoprotein encoded in said
- 20 baculovirus.
6. The insect cell according to claim 5 which is a Spodoptera frugiperda (Sf9) cell.
7. A baculovirus-expressed influenza A or influenza B virus nucleoprotein.
- 25 8. A method of producing a baculovirus-expressed influenza A or B virus nucleoprotein comprising culturing host cells according to claim 5, in a manner allowing the expression of said nucleoprotein, and isolating said nucleoprotein from said cells.
- 30 9. A bioassay for the diagnosis of influenza in mammals comprising the steps of:
 - i) coating a surface with the baculovirus-expressed nucleoprotein of claim 7;

ii) contacting said coated surface with a biological sample from a mammal suspected of having influenza; and

iii) detecting the presence or absence of a
5 complex formed between said protein and antibodies specific therefore present in said biological sample.

10 10. The bioassay according to claim 9 wherein said surface is a polystyrene microtitration plate, a slide or nylon or nitrocellulose membranes generated by immunoblot or Western blot.

15 11. A diagnostic kit comprising the baculovirus-expressed nucleoprotein according to claim 7 and ancillary reagents suitable for use in detecting the presence or absence of antibodies to said protein in a biological sample.

20 12. A vaccine for mammals against influenza A or influenza B virus comprising the baculovirus-expressed nucleoprotein according to claim 7, in an amount sufficient to induce immunization against said virus, and a pharmaceutically acceptable carrier.

13. The vaccine according to claim 12 which further comprises an adjuvant.

1/10

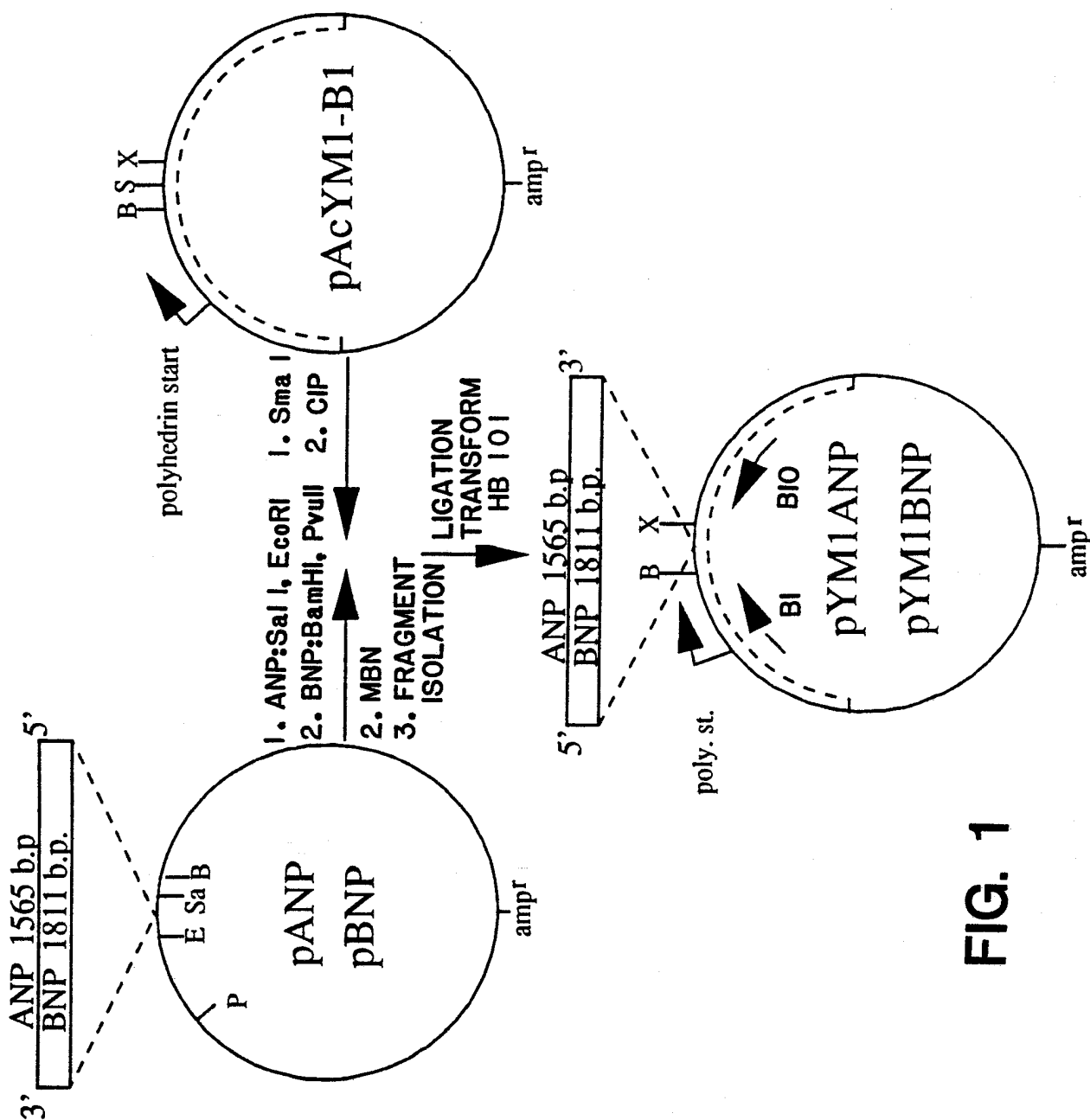


FIG. 1

FIG. 2B

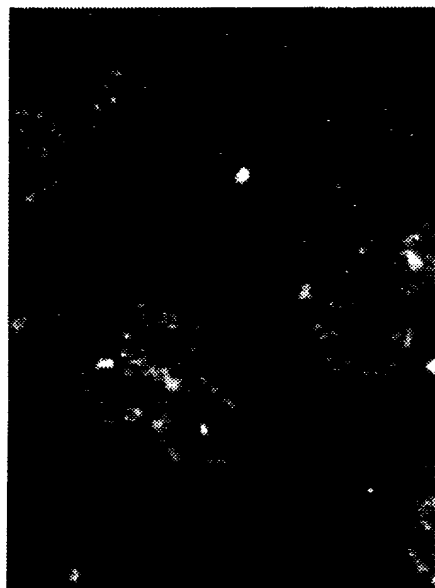


FIG. 2D



FIG. 2A

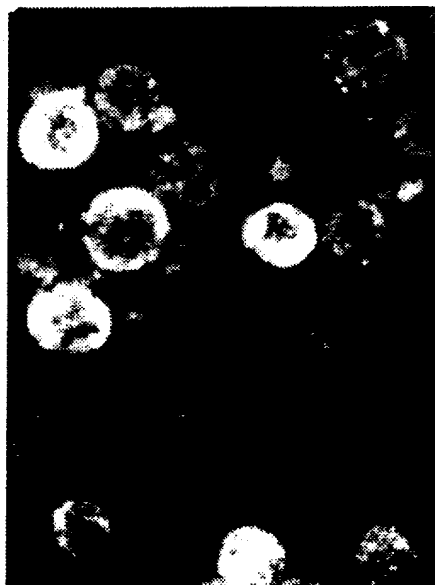
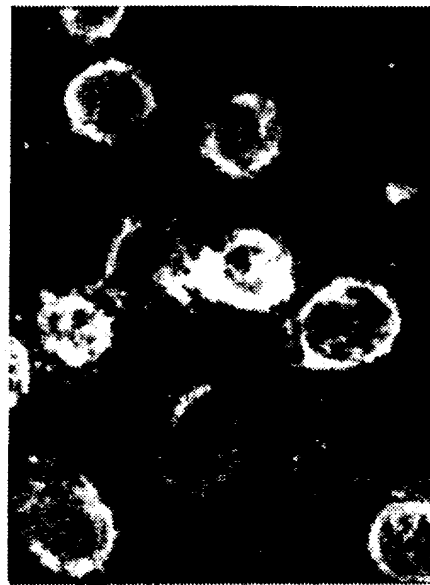


FIG. 2C



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FIG. 3A

1 2 3

FIG. 3B

1 2 3



FIG. 4A

1 2 3 4

FIG. 4B

1 2 3 4



FIG. 5A

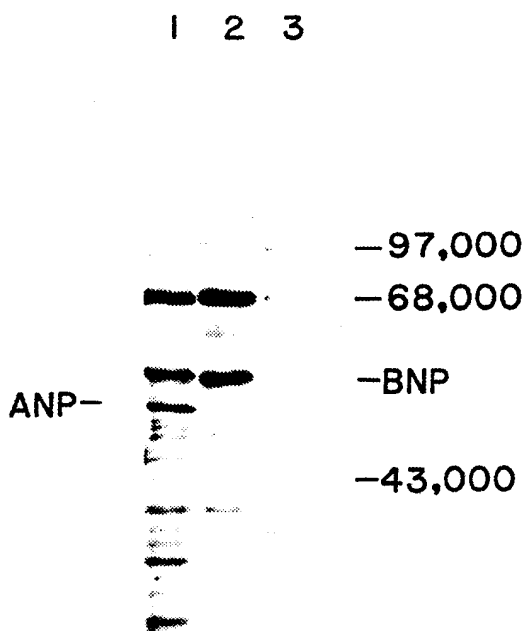


FIG. 5B

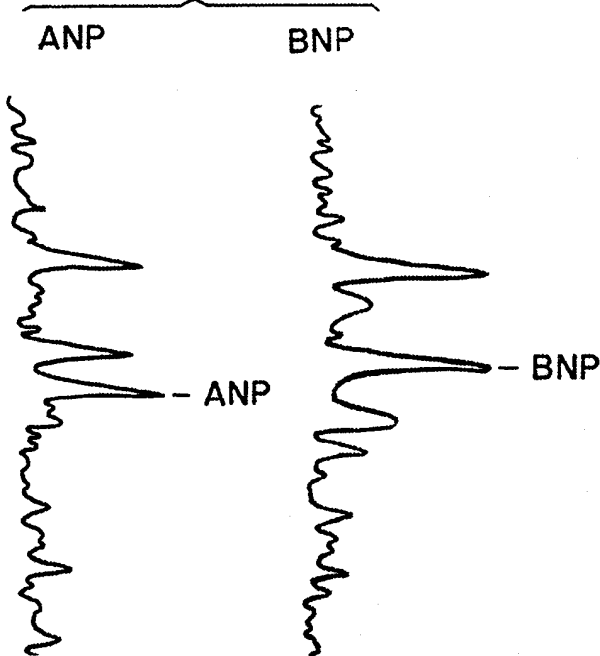


FIG. 9A

S1 S2



FIG. 9B

S1 S2



FIG. 9C

S1 S2



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ANP

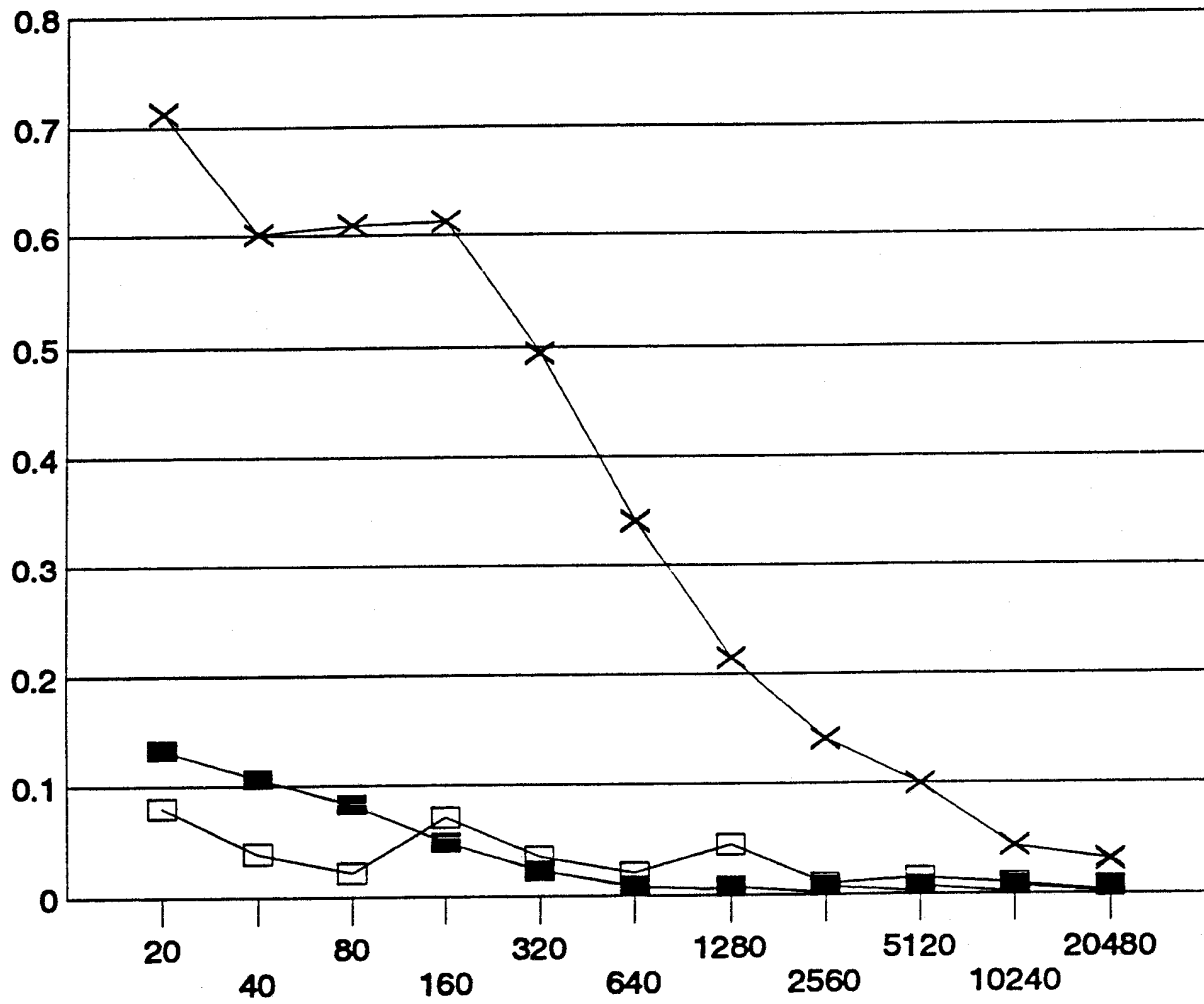


FIG. 6A

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BNP

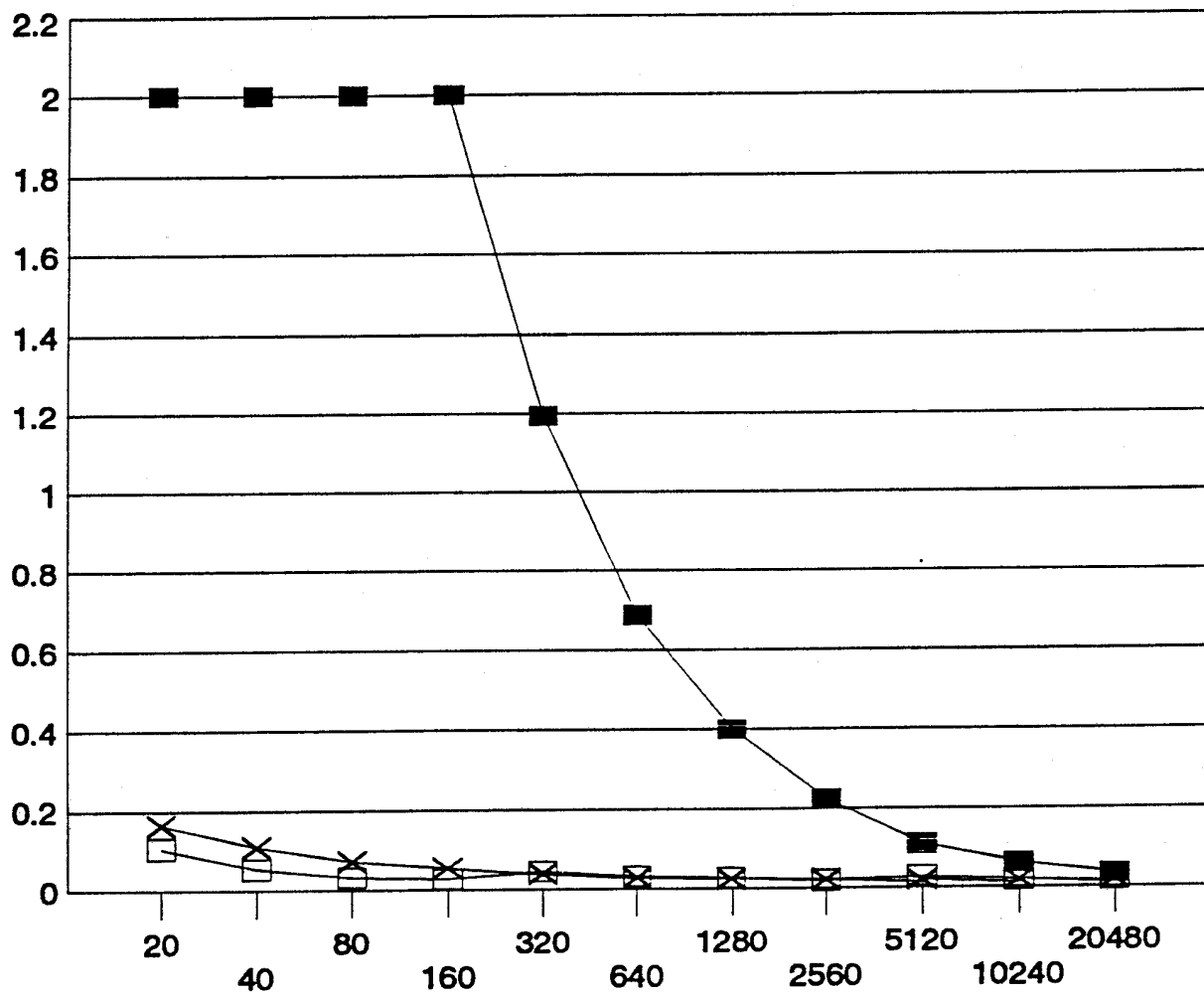


FIG. 6B

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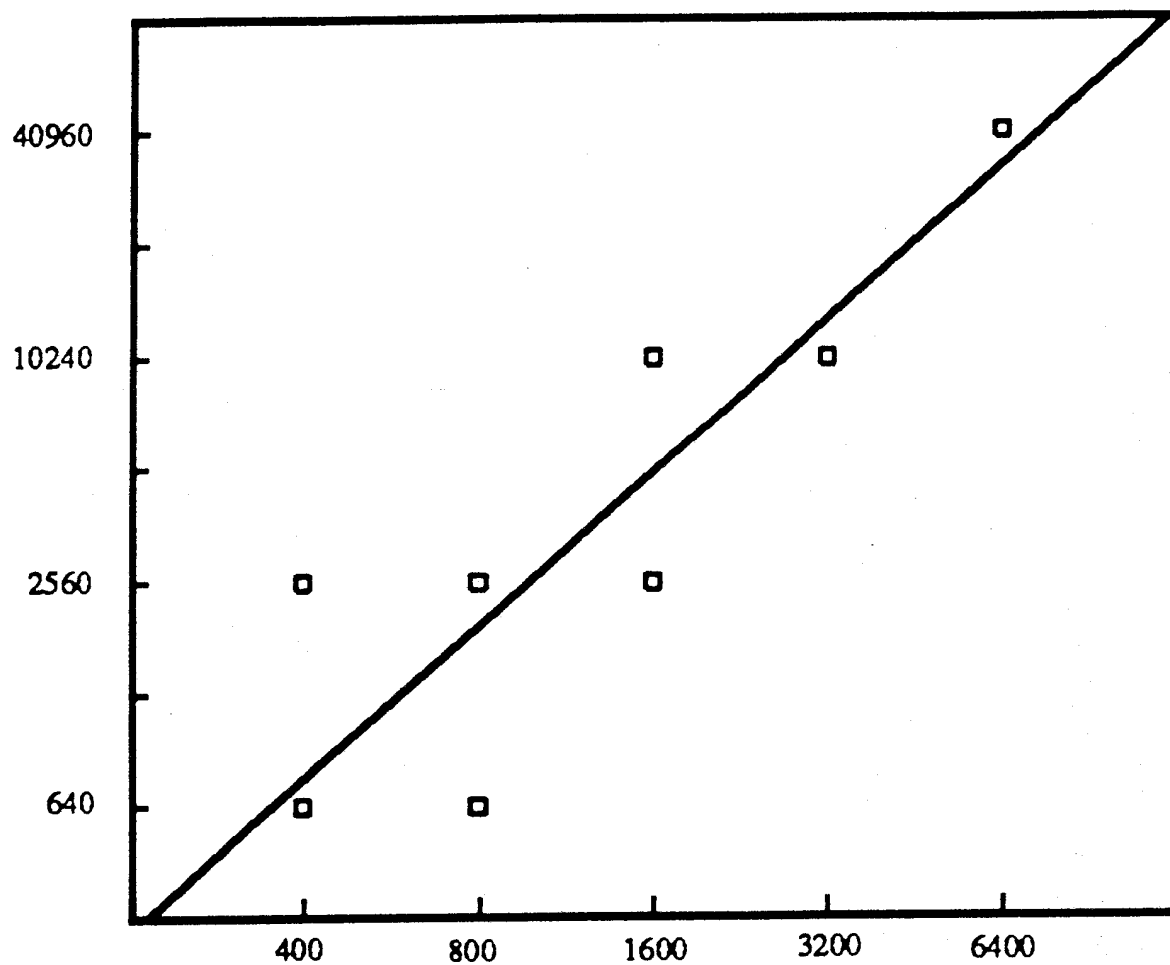


FIG. 7A

8 / 10

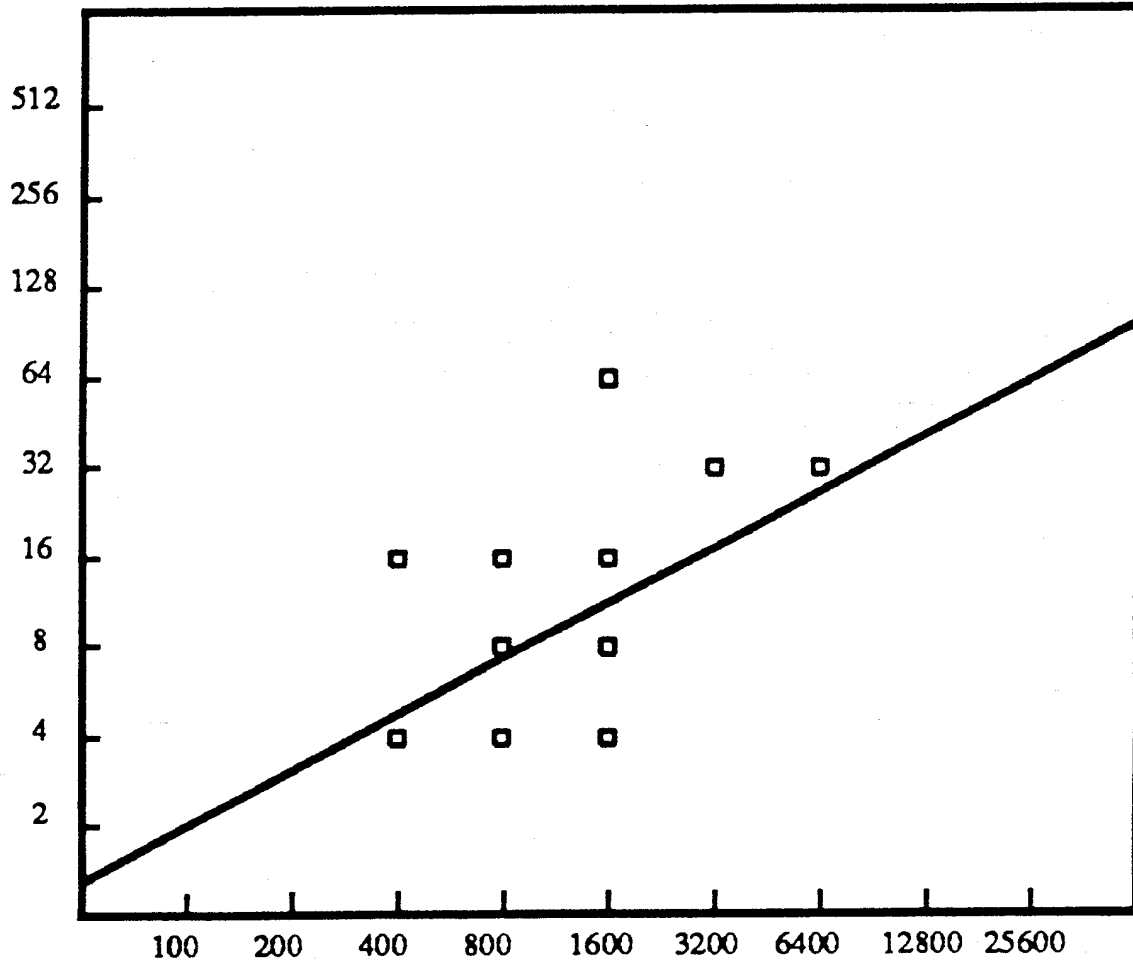


FIG. 7B

9 / 10

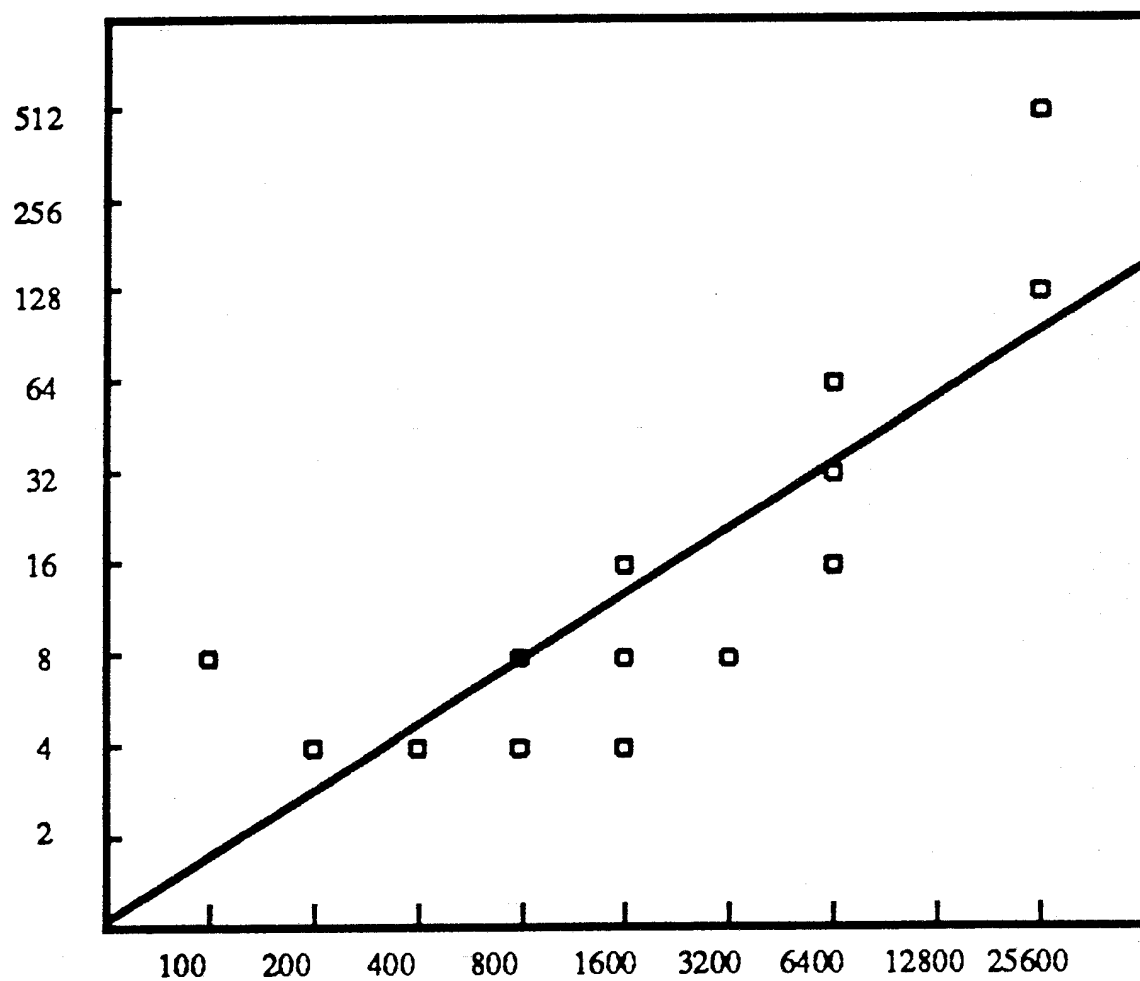


FIG. 7C

FIG. 8A

S1 S2

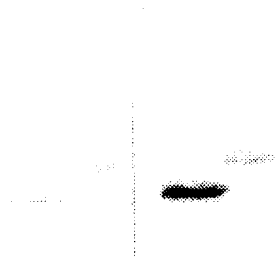


FIG. 8B

S1 S2

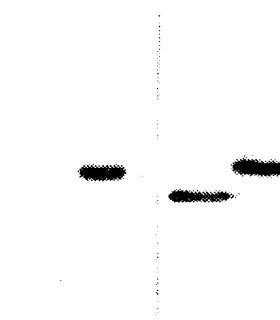


FIG. 8C

S1 S2

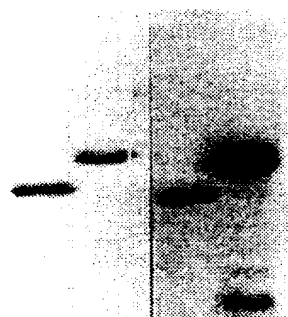
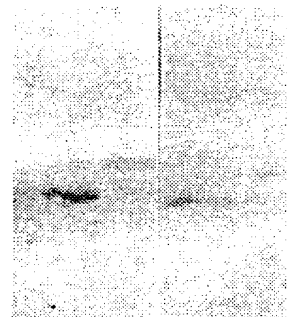


FIG. 8D

S1 S2



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02074

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³ According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): Please See Attached Sheet. US CL : 435/320.1, 69.3, 240.2; 424/89; 536/27; 530/350; 436/518; 206/223.																	
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched⁴</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; text-align: left; border-bottom: 1px solid black;">Classification System</th> <th style="text-align: left; border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">U.S.</td> <td style="border: 1px solid black; padding: 5px;">435/320.1, 69.3, 240.2; 424/89; 536/27; 530/350; 436/518; 206/223.</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched⁵</div> <p>APS, BIOSIS. Search terms: influenza, baculovirus, nucleoprotein, NP.</p>			Classification System	Classification Symbols	U.S.	435/320.1, 69.3, 240.2; 424/89; 536/27; 530/350; 436/518; 206/223.											
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U.S.	435/320.1, 69.3, 240.2; 424/89; 536/27; 530/350; 436/518; 206/223.																
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; text-align: left; border-bottom: 1px solid black;">Category*</th> <th style="text-align: left; border-bottom: 1px solid black;">Citation of Document,¹⁵ with indication, where appropriate, of the relevant passages¹⁷</th> <th style="width: 15%; text-align: left; border-bottom: 1px solid black;">Relevant to Claim No. ¹⁸</th> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top;">X/Y</td> <td style="border: 1px solid black; padding: 5px;">Journal of General Virology, Vol. 71, issued July 1990, Rota et al. "Expression of influenza A and B virus nucleoprotein antigens in baculovirus", pp. 1545-1554. See entire article.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top;">1-11/12,13</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top;">X/Y</td> <td style="border: 1px solid black; padding: 5px;">Archives of Virology, Vol. 113 Number 3-4, issued 8 October 1990, Harley et al. "Characterisation of an avian influenza virus nucleoprotein expressed in E. coli and in insect cells", pp. 267-277. See entire article.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top;">1-8/9-13</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top;">Y</td> <td style="border: 1px solid black; padding: 5px;">Journal of General Virology, Vol. 68, issued 1987, Matsuura et al. "Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins", pp. 1233-1250. See entire article.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top;">1-13</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top;">Y</td> <td style="border: 1px solid black; padding: 5px;">Virology, Vol. 167, issued 1988, Cox et al. "Identification of sequence changes in the cold-adapted live attenuated influenza vaccine strain, A/Ann Arbor/6/60 (H2N2)", pp. 554-567. See Fig. 3 especially.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top;">1-13</td> </tr> </table>			Category*	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸	X/Y	Journal of General Virology, Vol. 71, issued July 1990, Rota et al. "Expression of influenza A and B virus nucleoprotein antigens in baculovirus", pp. 1545-1554. See entire article.	1-11/12,13	X/Y	Archives of Virology, Vol. 113 Number 3-4, issued 8 October 1990, Harley et al. "Characterisation of an avian influenza virus nucleoprotein expressed in E. coli and in insect cells", pp. 267-277. See entire article.	1-8/9-13	Y	Journal of General Virology, Vol. 68, issued 1987, Matsuura et al. "Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins", pp. 1233-1250. See entire article.	1-13	Y	Virology, Vol. 167, issued 1988, Cox et al. "Identification of sequence changes in the cold-adapted live attenuated influenza vaccine strain, A/Ann Arbor/6/60 (H2N2)", pp. 554-567. See Fig. 3 especially.	1-13
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents:¹⁶</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div>																	
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search² <div style="text-align: center;">02 June 1992</div> </td> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report ² <div style="text-align: center; font-size: 1.2em;">17 JUN 1992</div> </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;"> International Searching Authority¹ <div style="text-align: center;">ISA/US</div> </td> <td style="border: 1px solid black; padding: 5px;"> Signature of Authorized Officer ²⁰ <div style="text-align: center;">PHILIP W. CARTER</div> </td> </tr> </table>			Date of the Actual Completion of the International Search ² <div style="text-align: center;">02 June 1992</div>	Date of Mailing of this International Search Report ² <div style="text-align: center; font-size: 1.2em;">17 JUN 1992</div>	International Searching Authority ¹ <div style="text-align: center;">ISA/US</div>	Signature of Authorized Officer ²⁰ <div style="text-align: center;">PHILIP W. CARTER</div>											
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FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Nucleic Acids Research, Vol. 17 No. 9, issued 1989, Rota "Sequence of a cDNA clone of the nucleoprotein gene of influenza B/Ann Arbor/1/86", p. 3595. See entire article.	1-13
Y	Journal of General Virology, Vol. 68, Wraith et al. "Purified influenza virus nucleoprotein protects mice from lethal infection", pp. 433-440. See entire article.	7, 9-13

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

1. ☐ Claim numbers __, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers __, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers __, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:
Please See Attached Sheet.

- ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. (Telephone Practice)
- ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
- ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
- ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	Journal of Medical Virology, Vol. 27, issued 1989, Harmon et al. "Immunoassay for serologic diagnosis of influenza type A using recombinant DNA produced nucleoprotein antigen and monoclonal antibody to human IgG", pp. 25-30. See entire article.	1-13
A	Virology, Vol. 154, issued 1986, Ryan et al. "Functional expression of influenza A viral nucleoprotein in cells transformed with cloned DNA", pp. 144-154. See entire article.	1-8

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

I. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12N 15/00, 15/33, 5/06; A61K 39/145; C07H 21/04, 13/00; G01N 33/543; B65D 71/00.

VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-6 and 8 drawn to a first product group consisting of DNA, baculovirus, and insect cells comprising DNA encoding a nucleoprotein of influenza A or B virus and baculovirus regulatory sequences; and to a method of using the first product group to produce influenza A or B virus nucleoprotein. Class 435 subclass 69.3

II. Claim 7 drawn to a second product, an influenza A or B virus nucleoprotein. Class 530 subclass 350.

III. Claims 9-10 drawn to a method of using the product of invention II, a nucleoprotein, to diagnose influenza in mammals. Class 436 subclass 518.

IV. Claim 11 drawn to an apparatus (kit) for performing the method of Invention III. Class 206 subclass 223.

V. Claims 12 and 13 drawn to a third product, a vaccine. Class 424 subclass 89.

The claims of these five groups are drawn to distinct inventions which are not linked so as to form a single general inventive concept. PCT Rules 13.1 and 13.2 do not provide for multiple products and methods.